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(71) Applicant: CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).

(72) Inventors: GERSHENGORN, Marvin, C.; 400 East 56th Street, New York, NY 10022 (US). ARVANITAKIS, Leandros; 536 East 79th Street, New York, NY 10021 (US). GERAS–RAAKA, Elizabeth; 34 Maplewood Avenue, Dobbs Ferry, NY 10522 (US). CESARMAN, Ethel; 820 Park Avenue, Hoboken, NJ 07030 (US).

(74) Agents: GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).

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(54) Title: CONSTITUTIVELY ACTIVE G PROTEIN COUPLED RECEPTOR OF HHV 8 AND METHOD

(57) Abstract

The present invention is directed to a constitutively active G protein coupled receptor of human herpesvirus 8, as well as a method of identifying negative antagonists of a constitutively active G protein coupled receptor. The method comprises co–expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein expression of the reporter protein is controlled by a promoter responsive to a signalling pathway activated by the constitutively active G protein coupled receptor; exposing the host cell to a test substance; and determining a level of reporter protein activity, wherein the level of reporter protein activity indicates effectiveness of the test substance as a negative antagonist of the constitutively active G protein coupled receptor. The invention further provides a method of preventing tumor formation or cell proliferation caused by a constitutively active G protein coupled receptor. This method comprises administering an amount of the negative antagonist so identified to a subject in an amount effective to prevent tumor formation or cell proliferation.

CONSTITUTIVELY ACTIVE G PROTEIN COUPLED RECEPTOR OF HHV 8 AND METHOD

5

FIELD OF THE INVENTION

The present invention relates to a
constitutively active G protein coupled receptor of human
10 herpesvirus 8. The invention further relates to a method
of identifying negative antagonists of constitutively
active G protein coupled receptors, and to a method of
preventing tumor formation or cell proliferation caused
by the constitutively active G protein coupled receptor
15 by administering an effective amount of the identified
negative antagonist.

BACKGROUND OF THE INVENTION

20   Throughout this application various
publications are referenced, many in parenthesis. Full
citations for these publications are provided at the end
of the Detailed Description of the Invention. The
disclosures of these publications in their entireties are
25 hereby incorporated by reference in this application.
          The family of guanine nucleotide-binding (G)
protein-coupled receptors (GPCRs) has been estimated to
be comprised of as many as 2,000 members, fully more than
1.5% of all the proteins encoded in the human genome,
30 that are thought to regulate function of virtually every
cell in the body. Furthermore, it has been estimated
that more than 50% of the drugs in use clinically in
humans at the present time are directed at GPCRs.

　　　　　In general, GPCRs require agonist binding for
activation. Constitutive (or agonist-independent)
signalling activity in mutant receptors has been well
documented but only a few GPCRs have been shown to
5　　exhibit agonist-independent activity in the wild type (or
native) form. For example, native dopamine D1B and
prostaglandin EP1b receptors have been found to possess
constitutive activity (Tiberi and Caron 1994; Hasegawa et
al. 1996). A number of GPCRs, for example, receptors for
10　thyroid-stimulating hormone (Vassart et al. 1995), have
been found to be mutated to exhibit agonist-independent
activity and cause disease in humans. Experimentally,
several single amino acid mutations have produced agonist
independent activity. β2 and α2 adrenergic receptors,
15　for example, mutated at single sites in the third
cytoplasmic loop show constitutive activity (Ren et al.
1993; Samama et al. 1994). In some cases, a large
deletion mutation in the carboxy tail or in the
intracellular loops of GPCRs has led to constitutive
20　activity. For example, in the thyrotropin releasing
hormone receptor a truncation deletion of the carboxyl
terminus renders the receptor constitutively active
(Nussenzveig et al. 1993; Matus-Leibovitch et al. 1995)
and a smaller deletion in the second extracellular loop
25　of the thrombin receptor causes constitutive activity
(Nanevicz et al. 1995) also.
　　　　　The finding that certain receptors exhibit
constitutive activity has led to a modification of
traditional receptor theory (Samama et al. 1993). It is
30　now thought that receptors can exist in at least two
conformations, an inactive conformation (R) and an
activated conformation (R*), and that an equilibrium
exists between these two states that markedly favors R
over R* in the majority of receptors. In some native
35　receptors and in the mutants described above, it has been

proposed that in the absence of agonist there is a shift
in equilibrium that allows a sufficient number of
receptors to be in the active R* state to initiate
signalling.

5   As many more GPCRs with constitutive activity
are found, both native as well as mutated receptors, a
newly recognized class of drugs termed negative
antagonists (or inverse agonists) will become important
therapeutic agents. Negative antagonism is demonstrated
10   when a drug binds to a receptor that exhibits
constitutive activity and reduces this activity.
Negative antagonists appear to act by constraining
receptors in an inactive state (Samama et al. 1994).
Although first described in other receptor systems
15   (Schutz and Freissmuth 1992), negative antagonism has
been shown to occur with guanine nucleotide-binding (G)
protein-coupled receptors (GPCRs), for example, opoid
(Costa and Herz 1989; Costa et al. 1992), β_2-adrenergic
(Samama et al. 1994; Pei et al. 1994; Chidiac et al.
20   1994), serotonin type 2C (Barker et al. 1994), bradykinin
(Leeb-Lundberg et al. 1994), and D1B dopamine receptors
(Tiberi and Caron 1994). Thus, a technology for
identifying negative antagonists of native and mutated
GPCRs has important predictable as well as not yet
25   realized pharmaceutical applications. Furthermore,
because constitutively active GPCRs are tumorigenic, the
identification of negative antagonists for these GPCRs
can lead to the development of anti-tumor and/or anti-
cell proliferation drugs.

30

                    SUMMARY OF THE INVENTION

            To this end, the subject invention provides a
constitutively active G protein coupled receptor of human
35   herpesvirus 8, and a method of identifying negative

antagonists of a constitutively active G protein coupled
receptor. The method comprises co-expressing in a host
cell a constitutively active G protein coupled receptor
and a reporter protein, wherein expression of the
5 reporter protein is controlled by a promoter responsive
to a signalling pathway activated by the constitutively
active G protein coupled receptor; exposing the host cell
to a test substance; and determining a level of reporter
protein activity, wherein the level of reporter protein
10 activity indicates effectiveness of the test substance as
a negative antagonist of the constitutively active G
protein coupled receptor.

          The invention further provides a method of
preventing tumor formation or cell proliferation in a
15 subject caused by a constitutively active G protein
coupled receptor. This method comprises identifying a
negative antagonist of a constitutively active G protein
coupled receptor; and administering to the subject an
amount of the negative antagonist effective to prevent
20 tumor formation or cell proliferation caused by the
constitutively active G protein coupled receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

25   These and other features and advantages of this
invention will be evident from the following detailed
description of preferred embodiments when read in
conjunction with the accompanying drawings in which:

          Fig. 1 illustrates the putative two-dimensional
30 topology of the HHV 8 GPCR;

          Figs. 2 and 3 illustrate competition binding of
human recombinant ^{125}I-IL-8 by unlabelled chemokines to
COS-1 cells transfected with pcKSHV GPCR (10 mg/ml). (2)
C-X-C chemokines (K_d or K_i): IL-8 (25 nM), NAP-2 (23 nM),
35 PF-4 (150 nM) and MGSA (270 nM). (3) C-C chemokines (K_d or

K_i): I-309 (110 nM) and RANTES (520 nM). Data represent
the mean of triplicate determinations in a representative
of two experiments;

Figs. 4 and 5 illustrate the characterization
of inositol phosphate (IP) accumulation in COS-1 cells
transfected with pcKSHV GPCR. (4) Comparison with COS-1
cells transfected with plasmids for human IL-8 receptors
(type A or B; 10 mg/ml) in the presence or absence of
plasmid encoding Ga_{16} (2 mg/ml) after 90 minutes. (5)
Time course in COS cells transfected with 10 mg/ml pcKSHV
GPCR. Data represent the mean +/- S.D. of triplicate
determinations in a representative of three experiments;
IP accumulation of mock-transfected, untreated cells has
been subtracted from all points;

Fig. 6 illustrates the dose dependence of
inositol phosphate (IP) accumulation in COS-1 cells after
transfection with various amounts of pcKSHV GPCR (from
0.003 to 10 mg/ml), measured at 90 min. Data represent
the mean +/- S.D. of triplicate determinations in a
representative of three experiments;

Fig. 7 illustrates the transcription of
luciferase driven by a PKC-responsive promoter in COS-1
cells transfected with pcKSHV GPCR. Data represent the
mean +/- S.D. of triplicate determinations in a
representative of three experiments; and

Fig. 8 illustrates cellular proliferation in
NRK-49F cells transfected with control plasmid or pcKSHV
GPCR. After 5 days in culture in medium supplemented
with calf serum or fetal calf serum, transfected cells
were trypsinized and counted. Cell counts; data
represent the mean +/- S.D. of triplicate culture dishes
of three transfections.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides a constitutively active G protein coupled receptor of human herpesvirus 8.

The invention further provides a method of identifying negative antagonists of a constitutively active G protein coupled receptor. The method comprises co-expressing in a host cell a constitutively active G protein coupled receptor and a reporter protein, wherein expression of the reporter protein is controlled by a promoter responsive to a signalling pathway activated by the constitutively active G protein coupled receptor; exposing the host cell to a test substance; and determining a level of reporter protein activity, wherein the level of reporter protein activity indicates effectiveness of the test substance as a negative antagonist of the constitutively active G protein coupled receptor.

The constitutively active G protein coupled receptor can be any such receptor which one desires to turn off. For example, GPCRs which are tumorigenic or which cause cells to proliferate could be of interest for application of the method of the subject invention. By identifying negative antagonists of such GPCRs, the negative antagonists could be used to turn off the GPCR and thereby eliminate the GPCR's tumorigenic or cell proliferative effects. An example of such a constitutively active GPCR is the GPCR of human herpesvirus 8 (HHV 8) (also known as Kaposi's sarcoma associated herpesvirus or KSHV) (Cesarman et al. 1996).

Having identified a GPCR of interest, the signalling pathway activated by the GPCR is identified and a promoter responsive to the signalling pathway is selected. Such a "responsive" promoter will only promote expression of a downstream gene if the GPCR is

functioning (i.e., signalling through its pathway).
GPCRs use various signalling pathways, including cyclic
AMP pathways and protein kinase C pathways. Suitable
promoters for use with such GPCRs are cyclic AMP-

5   responsive promoters and protein kinase C-responsive
promoters, respectively. For a review of the cyclic AMP
signalling pathway see Habener 1995, and for a review of
the protein kinase C signalling pathway see Gershengorn
and Perlman 1995.

10   A reporter protein is then chosen. The
activity levels of the reporter protein will indicate the
levels of GPCR signalling, since the reporter protein
will only be expressed if the GPCR is signalling through
its pathway to which the selected promoter is responsive.

15   A reporter protein should be chosen which is not generaly
expressed in the host cell to be utilized for co-
expression, to eliminate any false indications of GPCR
signalling. A suitable reporter protein, as disclosed in
the following examples, is the firefly luciferase gene.

20   The activity of this reporter protein can be readily
assayed using a luminescent assay. Other reporter
proteins could also be readily used in the method of the
subject invention, such as β-galactosidase and growth
hormone.

25   Various host cells can be utilzed in the method
of the subject invention. The choice of a host cell is
based on several factors, including the absence of
endogenous expression of the reporter protein in the host
cell, presence of the GPCR signalling pathway in the host

30   cell, and capability of co-expressing the GPCR and the
reporter protein in the host cell. For the analysis of
human GPCRs, mammalian cells are preferred, such as COS
cells.

          Generally the co-expression of the

35   constitutively active G protein coupled receptor and the

reporter protein in the host cell will involve
introduction of two constructs into the host cell. The
first construct is a nucleic acid molecule encoding the G
protein coupled receptor under the control of suitable
5   regulatory molecules for expression of the GPCR in the
host cell. The second construct is a nucleic acid
molecule encoding the reporter protein under the control
of the promoter responsive to the signalling pathway of
the GPCR. Each construct can be provided in the form of
10   a plasmid, or a single plasmid comprising both constructs
could be used. Techniques for introducing the
construct(s) into the host cells may involve the use of
expression vectors (such as plasmids and viruses) which
comprise the nucleic acid molecule encoding the
15   construct(s). DNA encoding the construct can be injected
into the nucleus of a host cell or transformed into the
host cell using a suitable vector, or mRNA encoding the
construct can be injected directly into the host cell, in
order to obtain expression of the GPCR and the reporter
20   protein in the host cell.
          Various methods are known in the art for
introducing nucleic acid molecules into host cells. One
method is microinjection, in which DNA is injected
directly into the nucleus of cells through fine glass
25   needles (or RNA is injected directly into the cytoplasm
of cells). Alternatively, DNA can be incubated with an
inert carbohydrate polymer (e.g. dextran) to which a
positively charged chemical group (e.g. diethylaminoethyl
("DEAE")) has been coupled. The DNA sticks to the DEAE-
30   dextran via its negatively charged phosphate groups.
These large DNA-containing particles, in turn, stick to
the surfaces of cells, which are thought to take them in
by a process known as endocytosis. Some of the DNA
evades destruction in the cytoplasm of the cell and
35   escapes to the nucleus, where it can be transcribed into

RNA like any other gene in the cell. In another method,
cells efficiently take in DNA in the form of a
precipitate with calcium phosphate. In electroporation,
cells are placed in a solution containing DNA and

5   subjected to a brief electrical pulse that causes holes
to open transiently in their membranes. DNA enters
through the holes directly into the cytoplasm, bypassing
the endocytotic vesicles through which they pass in the
DEAE-dextran and calcium phosphate procedures (passage

10 through these vesicles may sometimes destroy or damage
DNA). DNA can also be incorporated into artificial lipid
vesicles, liposomes, which fuse with the cell membrane,
delivering their contents directly into the cytoplasm.
In an even more direct approach, DNA is absorbed to the

15 surface of tungsten microprojectiles and fired into cells
with a device resembling a shotgun.

          Further methods for introducing nucleic acid
molecules into cells involve the use of viral vectors.
Since viral growth depends on the ability to get the

20 viral genome into cells, viruses have devised clever and
efficient methods for doing it. Various viral vectors
have been used to transform mammalian cells, such as
vaccinia virus, adenovirus, and retrovirus.

          As indicated, some of these methods of

25 transforming a cell require the use of an intermediate
plasmid vector. U.S. Patent No. 4,237,224 to Cohen and
Boyer describes the production of expression systems in
the form of recombinant plasmids using restriction enzyme
cleavage and ligation with DNA ligase. These recombinant

30 plasmids are then introduced by means of transformation
and replicated in unicellular cultures including
procaryotic organisms and eucaryotic cells grown in
tissue culture. The DNA sequences are cloned into the
plasmid vector using standard cloning procedures known in

35 the art, as described by Sambrook et al. (1989).

          Various test substances can be screened for
their effectiveness as negative antagonists of a
constitutively active GPCR. Test substances can be, for
example, traditional chemical compounds (such as
5   benzodiazepines) or peptides. Any suitable test
substance can be screened in the subject method, as long
as the host cell can be exposed to the test substance.
          Having thus identified negative antagonists of
a constitutively active G protein coupled receptor, and
10 knowing that constitutively active G protein coupled
receptors cause tumor formation or cell proliferation,
the invention further provides a method of preventing
tumor formation or cell proliferation in a subject caused
by a constitutively active G protein coupled receptor.
15 The method comprises identifying a negative antagonist of
a constitutively active G protein coupled receptor (as
discussed above); and administering to the subject an
amount of the negative antagonist effective to prevent
tumor formation or cell proliferation caused by the
20 constitutively active G protein coupled receptor. In one
embodiment, the tumor formation or cell proliferation is
caused by a constitutively active G protein coupled
receptor of a virus such as human herpesvirus 8.
          The following examples relate to the
25 identification of a constitutively active GPCR of human
herpesvirus 8, and the assaying of the GPCR activity by
co-expressing a reporter protein under the control of a
promoter responsive to the GPCR signalling pathway. By
then exposing the host cell to a test substance, the
30 effectiveness of the test substance as a negative
antagonist of the constitutively active GPCR can be
determined (by determining the effect of the test
substance on reporter protein activity). The identified
negative antagonists could serve as probes to determine
35 the role of agonist-independent HHV8 GPCR activity in

diseases associated with HHV 8 infections or as lead
compounds for drugs (Navia and Murcko 1992) if a role for
HHV8 GPCR in a disease process(es) is demonstrated. In
particular, because constitutively active GPCRs have been
5   found to be tumorigenic in mice (Young et al. 1986) or to
mediate proliferation or transformation of cells infected
with HHV 8 (Julius et al. 1989; Allen et al. 1991;
Gutkind et al. 1991), these negative antagonists could
lead to the development of anti-tumor drugs.

10

MATERIALS AND METHODS

            Competition binding. The coding sequence of
KSHV GPCR was cleaved from its original subcloning
15   plasmid (Cesarman et al. 1996) by digestion with NcoI and
inserted into the EcoRV site of the eukaryotic expression
vector pcDNA3.1(+) (Invitrogen), producing the pcKSHV
GPCR used for expression in mammalian cells. COS-1 cells
(American Type Culture Collection) were grown in 100 mm
20   petri dishes in Dulbecco's modified Eagle's medium (DMEM;
GIBCO-BRL) with 5% Nu-Serum (Collaborative Research) in a
humidified incubator in an atmosphere of 5% CO_2 at 37°C.
DEAE-dextran transfections were carried out as previously
described (Nussenzveig et al. 1994). COS-1 cells were
25   transfected with 10 mg/ml of pcKSHV GPCR and tested for
IL-8 binding 48 hrs post-transfection. Approximately
100,000 cells per well (in 12-well plates) were washed
with Hank's balanced salt solution (HBSS) containing 25
mM HEPES, pH 7.4 (GIBCO-BRL). Between 0.3 and 0.5 nM
30   ^{125}I-IL-8 (DuPont-NEN) in the absence or presence of
various concentrations of unlabelled chemokines was added
and binding was allowed to proceed at 4°C for 2 hrs with
gentle rocking. The binding buffer contained bovine
serum albumin (BSA; 1 mg/ml), bacitracin (1 mg/ml), and
35   phenyl methyl sulphonyl fluoride (PMSF; 1mM). Assays

were terminated by aspirating the binding buffer, washing
three times with chilled HBSS and solubilizing the cells
with 0.4 N NaOH. An aliquot of this solution was counted
in a gamma counter and the data expressed as percent of
5   maximum bound. Binding and dose-response curves were
analyzed using Prism software (GraphPad, Inc.).

        Inositol phosphate accumulation. COS-1 cells
were transfected with pcKSHV GPCR or plasmids encoding
human IL-8 receptor types A or B and either Ga_{16} or Ga_{15}
10   (Wu et al. 1993). All transfection cocktails were
supplemented to contain equal total plasmid DNA by the
addition of appropriate amounts of vector (pcDNA3.1).
Twenty four hrs following transfection, cells were
harvested and reseeded in 12-well plates in DMEM with 10
15   % Nu serum and 1 mCi/ml myo-$[^3H]$ inositol (DuPont-NEN), and
maintained in culture as before for an additional 24 hrs.
After removing the culture media and rinsing the cells,
cells were incubated in HBSS containing 25 mM HEPES, pH
7.4 with 10 mM LiCl in the absence or presence of human
20   recombinant IL-8 (R&D Systems) at 0.25 mg/ml. Cells were
harvested after 90 min or other time points, and IP
accumulation measured using ion exchange chromatography
as described previously (Straub et al. 1990).

        Luciferase assay. Cells were transfected with
25   increasing amounts of pcKSHV-GPCR (0.003 to 0.100 mg/ml)
and 5 mg/ml pAP1-fos-LUC (Schadlow et al. 1992),
supplemented to contain equal total plasmid DNA by the
addition of appropriate amounts of vector (pcDNA3.1).
Cells were washed with phosphate-buffered saline (PBS)
30   and lysed with 0.5 ml/10 cm dish of lysis buffer (25 mM
GlyGly, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol (DTT),
1% Triton X-100, pH 7.8). Cell extracts were diluted
1:10 or 1:20 in reaction buffer (25 mM GlyGly, 15 mM
$MgSO_4$, 4 mM EGTA, 1 mM DTT, 15 mM KH_2PO_4, 2 mM ATP, pH

7.8), combined with equal volumes of 0.4 mM luciferin in
reaction buffer and luminescence measured for 10 sec.

      Cell proliferation assay. "Normal rat kidney"
fibroblasts (NRK-49F; ATCC) were seeded at $2x10^5$ / 100 mm
dish, grown in DMEM with 10% fetal calf serum, and
transfected with control vector (20 mg/plate), pcKSHV
GPCR (20 mg/plate) or pRas-Zip (10 mg/plate; positive
control)(Dotto et al. 1985) using the calcium phosphate
method as previously described (Loyter et al. 1982).
Cells were harvested 24 hrs post-transfection and seeded
at a density of 5,000 cells / 100 mm dish in DMEM
supplemented with either 10 % calf serum or fetal calf
serum. On day 5 post-transfection, cells were
photographed for qualitative assessment (Nikon inverted
phase microscope) and then trypsinized to determine total
cell number using a hemocytometer.

EXAMPLE I

      Kaposi's sarcoma-associated herpesvirus
(KSHV)/human herpesvirus 8 (HHV 8) is a recently
described virus consistently present in Kaposi's sarcoma
(KS)(Chang et al. 1994; Moore and Chang 1995) and primary
effusion (body cavity-based) lymphomas (PEL)(Cesarman et
al. 1995) which are malignancies occurring frequently,
but not exclusively, in AIDS patients. KSHV is a
gammaherpesvirus with homology to Herpesvirus Saimiri
(HVS) and Epstein-Barr virus (EBV)(Chang et al. 1995;
Moore et al. 1996), both of which are viruses capable of
transforming lymphocytes (Roizman 1996). Cloning of a
viral genome fragment revealed the presence of an open
reading frame encoding a putative G protein-coupled
receptor (KSHV GPCR)(Cesarman et al. 1996) that is
homologous to a GPCR encoded by HVS (Nicholas et al.
1992; Ahuja and Murphy 1993) and to human interleukin 8

(IL-8) receptors (Holmes et al. 1991; Murphy and Tiffany
1991). In the studies that follow, it is shown that KSHV
GPCR is a *bona fide* signaling receptor with constitutive
(agonist-independent) activity via the

5   phosphoinositide-1,4,5 inositoltrisphosphate-protein
kinase C (PKC) cascade. Furthermore, KSHV GPCR is shown
to stimulate cellular proliferation, making it a
candidate viral oncogene. The nucleic acid and amino
acid sequences of the HHV 8 GPCR are disclosed in co-

10 pending, co-assigned U.S. Serial No. 08/728,603, filed
October 10, 1996, the contents of which are hereby
incorporated by reference.

            Fig. 1 illustrates the putative two-dimensional
topology of HHV 8 GPCR. The top of the diagram

15 represents the extracellular space (E), the middle
portion represents the transmembrane (TM) domain, and the
bottom portion represents the intracellular space (C).
Each circle represents an amino acid residue designated
by the single letter code.

20   As is evident, HHV 8 GPCR can be represented
with the signature seven TM-spanning domains of all
GPCRs. This, however, is not sufficient to classify this
protein as a receptor. Moreover, it is lacking some of
the residues that are highly conserved in GPCRs. The

25 most relevant to this application relate to amino acid
residues that have been found to be involved in
activation of other GPCRs. For example, an Asp residue
is usually present in putative TM-2 that has been shown
to be critical for activation of a number of GPCRs

30 (Baldwin 1994). There is an Asp at this position in both
IL8-Rs but not in the GPCR in the Herpesvirus saimiri
genome and not in HHV 8 GPCR. In HHV 8 GPCR this Asp has
been replaced by Asn. A similar replacement of Asp by
Asn in TM-2 is present in the receptor for

35 gonadotropin-releasing hormone (GnRH) but in the GnRH

receptor there is a complementary change of a highly
conserved Asn in TM-7, which is in a Asn-Pro-Xaa-Xaa-Tyr
motif (Baldwin 1993), to Asp (Zhou et al. 1994) that is
not present in HHV 8 GPCR. It has been postulated that
5   interactions between these two residues, Asp and Asn, may
be necessary for receptor activation (Van Rhee and
Jacobson 1996). There is a Val at this position in TM-7
in HHV 8 GPCR that will not be able to form a hydrogen
bond with the Asn in TM-2. The absence of this potential
10   interaction between TM-2 and TM-7 may render HHV 8 GPCR
constitutively active, but this cannot be predicted from
the sequence.

         A number of experiments were performed to show
that HHV 8 GPCR is a functioning receptor. To
15   investigate whether the KSHV GPCR gene encodes a
functional receptor, the KSHV GPCR was expressed in COS
cells and a determination whether the receptor binds IL-8
and other chemokines was made. IL-8, which is a member
of the family of C-X-C chemokines (Baggiolini et al.
20   1994), was chosen as radioligand because of the homology
of KSHV GPCR to cellular IL-8 receptors. Furthermore,
since KS lesions are sites of active inflammation and
have been shown to produce IL-8 and other cytokines
(Sciacca et al. 1994), it is possible that such areas
25   could provide stimulatory signals for the KSHV GPCR. The
binding characteristics of KSHV GPCR were investigated
using ^{125}I-IL-8 and various concentrations of other
chemokines in a competition analysis. As shown in Fig.
2, KSHV GPCR binds IL-8 with an apparent equilibrium
30   dissociation constant (K_d) of 30 nM, which is lower
affinity than the values previously reported for binding
of IL-8 to the human IL-8 receptor types A or B (K_d of
0.8-1.0 nM)(Holmes et al. 1991; Murphy and Tiffany 1991).
Since chemokine receptors may bind more than one ligand,
35   several other C-X-C chemokines were tested, namely MGSA,

NAP-2 and PF-4 (Baggiolini et al. 1994), and it was found
that they also bind to KSHV GPCR (Fig. 2). Although
chemokines of the C-C family do not bind to IL-8
receptors (Baggiolini et al. 1994), it was found that
5   I-309 and RANTES bind to KSHV GPCR whereas MIP-1b and
MCP-1 do not (Fig. 3). The rank order of binding was:
IL-8 = NAP-2 > PF-4 = I-309 = MGSA > RANTES >> MIP-1b and
MCP-1. Thus, KSHV GPCR exhibits binding characteristics
of a chemokine receptor with affinity for a range of
10   chemokines spanning the C-X-C and C-C families.

        To assess whether KSHV GPCR is a signal
transducing receptor, the formation of intracellular
second messengers in COS cells transiently expressing
KSHV GPCR was measured. Inositol phosphate (IP)
15   accumulation was measured because IL-8 receptors signal
via the phosphoinositide-IP-calcium/diacylglycerol-PKC
cascade (Holmes et al. 1991; Murphy and Tiffany 1991).
Stimulated IP formation can be measured in COS cells
co-expressing IL-8 receptors and the α subunits of the
20   heterotrimeric proteins G_{16} ($G\alpha_{16}$) or G_{15} ($G\alpha_{15}$) in the
presence but not in the absence of IL-8 (Wu et al. 1993).
In agreement with that report, little measurable
accumulation of IPs in COS cells expressing IL-8
receptors in the absence or presence of IL-8 was found,
25   unless $G\alpha_{16}$ or $G\alpha_{15}$ was cotransfected (Fig. 4). In cells
expressing IL-8 receptors and $G\alpha_{16}$ or $G\alpha_{15}$, IL-8 stimulated
IP formation. In contrast, and under these experimental
conditions, cells transiently expressing KSHV GPCR showed
IP accumulation which was higher than that obtained with
30   human IL-8 receptors activated by IL-8. In cells
transiently expressing KSHV GPCR, IP formation was
elevated in the absence of added ligand and without
co-expression of $G\alpha_{16}$ or $G\alpha_{15}$, and was not increased by
IL-8 or any of the chemokines that showed binding to KSHV
35   GPCR. There was a constant rate of IP accumulation for

at least 4 hours (Fig. 5); IL-8 had no effect at any time
point. Transfection of COS-1 cells with increasing
amounts of plasmid encoding KSHV GPCR showed that IP
accumulation was directly proportional to the amount of
5   plasmid used for transfection (between 0.1 and 3 mg/ml
plasmid) (Fig. 6). Again, there was no effect of IL-8 on
IP accumulation at any level of KSHV GPCR expression.
Thus, KSHV GPCR exhibits agonist-independent (or
constitutive) activity in COS-1 cells. In contrast to
10 IL-8 receptors, KSHV GPCR couples effectively to the G
protein(s) endogenous in COS-1 cells. Since IP
accumulation from the KSHV GPCR is resistant to pertussis
toxin, this suggests that the receptor may signal through
the Gq family of G-proteins.

15   To determine whether the constitutive signaling
activity of KSHV GPCR leads to stimulation of downstream
processes, the effect of basal second messenger formation
on gene transcription was measured. A reporter gene,
firefly luciferase, under the control of a PKC-responsive
20 promoter containing an AP-1 binding motif (Schadlow et
al. 1992) was used to measure activity of the
diacylglycerol/PKC limb of the phosphoinositide signaling
pathway. In these experiments, COS-1 cells were
co-transfected with plasmid encoding KSHV GPCR and a
25 plasmid containing a minimal promoter derived from the
human c-fos gene to which a PKC-responsive element was
inserted to drive luciferase transcription (Schadlow et
al. 1992). As shown in Fig. 7, there was a direct
relationship between the amount of plasmid encoding KSHV
30 GPCR used for transfection and the level of luciferase
activity. Thus, second messenger formation caused by the
basal activity of KSHV GPCR is sufficient to induce gene
transcription for a PKC-responsive promoter. This
finding is important given the wide range of genes,
35 including those for inflammatory and angiogenic agents,

whose transcription is regulated by PKC-responsive
promoters (Karin 1995). Of note is the observation that
luciferase activity (Fig. 7) is stimulated at lower
plasmid levels than IP accumulation (Fig. 6). This
5   likely occurred because induction of gene transcription
is a more amplified response occurring downstream of
second messenger formation.

                              EXAMPLE II
10
           To test whether the constitutive KSHV GPCR
signaling activity affects cellular proliferation,
NRK-49F cells were transiently transfected with plasmid
encoding KSHV GPCR and monitored for proliferation as
15   compared to cells transfected with control vector
(negative control) or with a plasmid encoding the Ras
oncogene (positive control) (Dotto et al. 1985). Culture
dishes transfected with Ras-encoding plasmids contained
more cells than control dishes within 24 to 48 hrs. As
20   early as 72 hrs post-transfection, the KSHV GPCR
transfectants exhibited increased cell number as compared
to the control transfectants. This response was
confirmed by phase contrast microscopy and quantified
after 5 days in culture (Fig. 8). All clusters
25   transfected with pcKSHV GPCR contained many more, closely
apposed cells than clusters of cells transfected with
control plasmid.
           Since gammaherpesviruses cause tranformation
(Roisman 1996) and GPCRs have been found to be oncogenic
30   under some circumstances (Julius et al. 1989), it is
possible that KSHV may be a transforming agent that acts,
at least in part, through KSHV GPCR. This possibility is
further strengthened by the specific association of KSHV
with KS and PEL, and the fact that KSHV GPCR is expressed
35   at the RNA level in both of these types of malignancies

(Cesarman et al. 1996; Arvanitakis et al. 1996). These
results have now demonstrated that KSHV GPCR is a *bona
fide* receptor that signals constitutively via the
phosphoinositide transduction pathway. Although it has
5   an amino acid sequence that is homologous to IL-8
receptors, binds IL-8 with relatively high affinity and
activates the same signal transduction cascade, KSHV GPCR
has two characteristics that make it a more promiscuous
receptor and one more likely to be involved in
10   pathogenesis than IL-8 receptors. KSHV GPCR signals
constitutively, that is, it does not require activation
by an agonist(s) and is capable of coupling to a wider
variety of G proteins than IL-8 receptors. Coupling of
KSHV GPCR to G proteins other than Ga_{16}, as shown by
15   signaling in COS cells that do not express this G
protein, would allow KSHV GPCR to signal in a broader
range of cell types. Constitutive signaling activity,
which is exhibited by some native GPCRs (Milano et al.
1994), has been observed more commonly with mutated GPCRs
20   (Lefkowitz et al. 1993; Alblas et al. 1996) in an
increasing number of human diseases, including tumors
(Coughlin 1994; Sande et al. 1995). Finally, the
enhanced proliferation exhibited by KSHV GPCR
transfectants supports a role for this receptor in
25   tumorigenesis.
          The presence of this functional, constitutively
active GPCR in the genome of a virus that is so tightly
associated with specific malignancies provides
circumstantial evidence toward its pathogenetic role.
30   Two alternative, or perhaps complementary, hypotheses can
be proposed to explain the role of the viral GPCR in the
development of KSHV-associated diseases: a direct
mechanism, in which the constitutive signaling activity
of KSHV GPCR leads to altered growth and neoplastic
35   transformation, and/or an indirect mechanism, where

signaling by KSHV GPCR induces the expression of other
genes which are in turn involved in pathogenetic events.
For example, the latter model could apply to the case of
basic fibroblast growth factor (bFGF) which has
5   previously been shown to play a pivotal role in the
development of KS lesions (Ensoli et al. 1989), and whose
expression is driven by an AP-1 binding, and probably
PKC-responsive promoter (Erdos et al. 1995).

        The determination of the effect on cells
10   expressing HHV 8 GPCRs of negative antagonists of HHV 8
GPCRs will delineate the role(s) of HHV 8 GPCRs in
infected cells. In this regard, the two most important
downstream effects are the stimulation of cell
proliferation or if HHV 8 GPCR activity caused cell
15   transformation. One can determine whether HHV 8 GPCR can
induce cell transformation and whether antagonists can
inhibit this effect. Transformation in NIH 3T3
fibroblasts can be used in a protocol used to show that
the agonist-activated serotonin 1c receptor, another
20   GPCR, is capable of triggering transformation (Julius et
al. 1989). In this assay, HHV 8 GPCR expression will be
produced by transfection with plasmids encoding HHV 8
GPCR, not by infection with HHV-8. This assay will show
whether HHV 8 GPCR expression leads to a transformed
25   phenotype and provide a quantitative assessment of the
effect of antagonist on transforming activity of HHV 8
GPCR because the number of foci of transformed cells in
the absence versus the presence of antagonist can be
counted. One can also determine whether HHV-8 infection
30   will transform CD19⁺ B-cells in experiments similar to
those performed to show lymphoblastoid transformation by
Herpesvirus saimiri and Epstein-Barr viruses (Rangan et
al. 1977; Miller 1974). If this series of experiments
shows that HHV 8 GPCR expression causes transformation in
35   these *in vitro* assays, one can determine whether cells

from these foci will generate tumors in nude mice and
whether this can be inhibited by antagonists.

      B-cell lines will be used for studies of cell
proliferation because of ease of their experimental
5   manipulation. These B-cell lines may not be good models
for study of proliferation because they may have acquired
other changes (mutations) that allow them to proliferate
without the signalling activity of HHV 8 GPCRs. Acutely
infected cells, such as B-cells, will be used and one can
10 study other cell lines including human lymphoblastoid
cell lines UH-1 and CB-33, and endothelial and fibroblast
cell lines derived from KS lesions. An effect of
antagonists of HHV 8 GPCR to inhibit the rates of cell
proliferation will be monitored. This will be tested in
15 a standard assay by measuring the rate of proliferation
of cells incubated without (control) and with
antagonists. By counting cells, which are seeded at low
density, over time, one can determine the effect of these
antagonists on the generation times of these cells.
20 Different incubation mediums can be tested and, in
particular, attempt to adjust the type and concentration
of the serum supplement so that there is a moderate
generation time for cells in the absence of antagonists.
This will allow for the highest likelihood of observing
25 an inhibitory effect on proliferation. These experiments
will be complemented with those that measure DNA
synthesis more directly, for example, by measuring
[^{3}H]thymidine incorporation into DNA and by measuring DNA
content, for example with 5-bromodeoxyuridine. The most
30 definitive measurement of cell proliferation is, however,
the rate of increase in cell number over time.

      These experiments will establish whether HHV 8
GPCR is expressed at the protein level in infected cells,
whether HHV 8 GPCR stimulates cellular transformation
35 and/or proliferation and whether the negative antagonists

discovered according to the method of the subject
invention will inhibit these effects.

        Although preferred embodiments have been
depicted and described in detail herein, it will be
5 apparent to those skilled in the relevant art that
various modifications, additions, substitutions and the
like can be made without departing from the spirit of the
invention and these are therefore considered to be within
the scope of the invention as defined in the claims which
10 follow.

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                          SEQUENCE LISTING

(1) GENERAL INFORMATION:

     (i) APPLICANT: Cornell Research Foundation, Inc.

    (ii) TITLE OF INVENTION: CONSTITUTIVELY ACTIVE G PROTEIN
                                COUPLED RECEPTOR OF HHV 8 AND
                                METHOD OF IDENTIFYING NEGATIVE
                                ANTAGONISTS OF G PROTEIN COUPLED
                                RECEPTORS

   (iii) NUMBER OF SEQUENCES: 1

    (iv) CORRESPONDENCE ADDRESS:
         (A) ADDRESSEE: Nixon, Hargrave, Devans & Doyle LLP
         (B) STREET: Clinton Square, P.O. Box 1051
         (C) CITY: Rochester
         (D) STATE: New York
         (E) COUNTRY: U.S.A.
         (F) ZIP: 14603

     (v) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
         (B) COMPUTER: IBM PC compatible
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
         (A) APPLICATION NUMBER:
         (B) FILING DATE:
         (C) CLASSIFICATION:

   (vii) PRIOR APPLICATION DATA:
         (A) APPLICATION NUMBER: US 08/785,928
         (B) FILING DATE: 22-JAN-1997

  (viii) ATTORNEY/AGENT INFORMATION:
         (A) NAME: Goldman, Michael L.
         (B) REGISTRATION NUMBER: 30,727
         (C) REFERENCE/DOCKET NUMBER: 19603/1321

    (ix) TELECOMMUNICATION INFORMATION:
         (A) TELEPHONE: (716) 263-1304
         (B) TELEFAX: (716) 263-1600

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 342 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ala Ala Glu Asp Phe Leu Thr Ile Phe Leu Asp Asp Asp Glu Ser
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Trp Asn Glu Thr Leu Asn Met Ser Gly Tyr Asp Tyr Ser Gly Asn Phe
            20   25   30

Ser Leu Glu Val Ser Val Cys Glu Met Thr Thr Val Val Pro Tyr Thr
        35   40   45

Trp Asn Val Gly Ile Leu Ser Leu Ile Phe Leu Ile Asn Val Leu Gly
    50   55   60

Asn Gly Leu Val Thr Tyr Ile Phe Cys Lys His Arg Ser Arg Ala Gly
65   70   75   80

Ala Ile Asp Ile Leu Leu Leu Gly Ile Cys Leu Asn Ser Leu Cys Leu
            85   90   95

Ser Ile Ser Leu Leu Ala Glu Val Leu Met Phe Leu Phe Pro Asn Ile
            100   105   110

Ile Ser Thr Gly Leu Cys Arg Leu Glu Ile Phe Phe Tyr Tyr Leu Tyr
        115   120   125

Val Tyr Leu Asp Ile Phe Ser Val Val Cys Val Ser Leu Val Arg Tyr
    130   135   140

Leu Leu Val Ala Tyr Ser Thr Arg Ser Trp Pro Lys Lys Gln Ser Leu
145   150   155   160

Gly Trp Val Leu Thr Ser Ala Ala Leu Leu Ile Ala Leu Val Leu Ser
            165   170   175

Gly Asp Ala Cys Arg His Arg Ser Arg Val Val Asp Pro Val Ser Lys
        180   185   190

Gln Ala Met Cys Tyr Glu Asn Ala Gly Asn Met Thr Ala Asp Trp Arg
        195   200   205

Leu His Val Arg Thr Val Ser Val Thr Ala Gly Phe Leu Leu Pro Leu
    210   215   220

Ala Leu Leu Ile Leu Phe Tyr Ala Leu Thr Trp Cys Val Val Arg Arg
225   230   235   240

Thr Lys Leu Gln Ala Arg Arg Lys Val Arg Gly Val Ile Val Ala Val
            245   250   255

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Val Leu Leu Phe Phe Val Phe Cys Phe Pro Tyr His Val Leu Asn Leu
            260             265             270

Leu Asp Thr Leu Leu Arg Arg Arg Trp Ile Arg Asp Ser Cys Tyr Thr
            275             280             285

Arg Gly Leu Ile Asn Val Gly Leu Ala Val Thr Ser Leu Leu Gln Ala
    290             295             300

Leu Tyr Ser Ala Val Val Pro Leu Ile Tyr Ser Cys Leu Gly Ser Leu
305             310             315             320

Phe Arg Gln Arg Met Tyr Gly Leu Phe Gln Ser Leu Arg Gln Ser Phe
            325             330             335

Met Ser Gly Ala Thr Thr
            340
```

WHAT IS CLAIMED IS:

1.   A method of identifying negative
antagonists of a constitutively active G protein coupled
receptor, said method comprising:

            co-expressing in a host cell a
constitutively active G protein coupled receptor and a
reporter protein, wherein expression of said reporter
protein is controlled by a promoter responsive to a
signalling pathway activated by the constitutively active
G protein coupled receptor;

            exposing said host cell to a test
substance; and

            determining a level of reporter protein
activity, wherein the level of reporter protein activity
indicates effectiveness of the test substance as a
negative antagonist of said constitutively active G
protein coupled receptor.

2.   The method of claim 1 wherein said
promoter is a cyclic AMP-responsive promoter.

3.   The method of claim 1 wherein said
promoter is a protein kinase C-responsive promoter.

4.   The method of claim 1 wherein said
reporter protein is luciferase.

5.   The method of claim 4 wherein determining
a level of luciferase activity uses a luminescent assay.

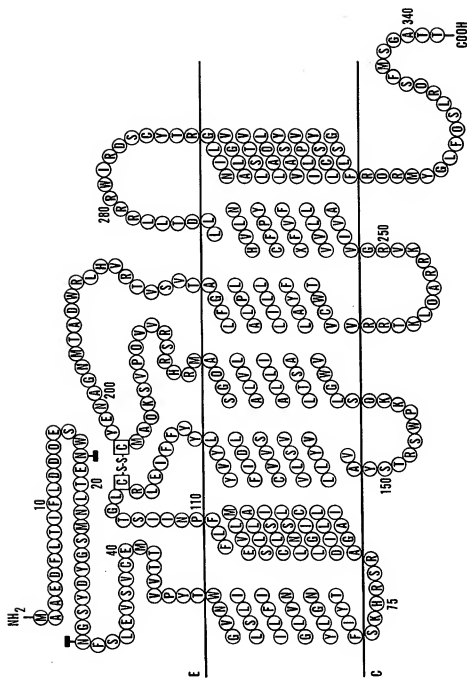6.   The method of claim 1 wherein said host
cell is a mammalian cell.

7.   The method of claim 6 wherein said
mammalian cell is a COS cell.

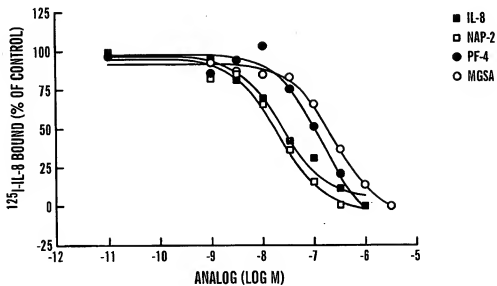8.   The method of claim 1 wherein said
constitutively active G protein coupled receptor is a G
protein coupled receptor of human herpesvirus 8.

9.   A method of preventing tumor formation or
cell proliferation in a subject caused by a
constitutively active G protein coupled receptor, said
method comprising:
                identifying a negative antagonist of a
constitutively active G protein coupled receptor; and
                administering to the subject an amount of
the negative antagonist effective to prevent tumor
formation or cell proliferation caused by the
constitutively active G protein coupled receptor.

10.   The method of claim 9 wherein said tumor
formation or cell proliferation is caused by a
constitutively active G protein coupled receptor of a
virus.

11.   The method of claim 10 wherein said virus
is a human herpesvirus 8.

12.   The method of claim 9 wherein said
identification of a negative antagonist of a
constitutively active G protein coupled receptor
comprises:
                co-expressing in a host cell a
constitutively active G protein coupled receptor and a
reporter protein, wherein expression of said reporter
protein is controlled by a promoter responsive to a

signalling pathway activated by the constitutively active
G protein coupled receptor;

        exposing said host cell to a test
substance; and

5   determining a level of reporter protein
activity, wherein the level of reporter protein activity
indicates effectiveness of the test substance as a
negative antagonist of said constitutively active G
protein coupled receptor.

10

        13.   The method of claim 12 wherein said
promoter is a cyclic AMP-responsive promoter.

        14.   The method of claim 12 wherein said
15 promoter is a protein kinase C-responsive promoter.

        15.   The method of claim 12 wherein said
reporter protein is luciferase.

20   16.   The method of claim 15 wherein determining
a level of luciferase activity uses a luminescent assay.

        17.   The method of claim 12 wherein said host
cell is a mammalian cell.

25

        18.   The method of claim 17 wherein said
mammalian cell is a COS cell.
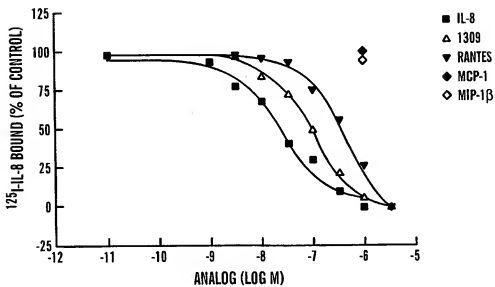

        19.   The method of claim 12 wherein said
30 constitutively active G protein coupled receptor is a G
protein coupled receptor of human herpesvirus 8.

        20.   A constitutively active G protein coupled
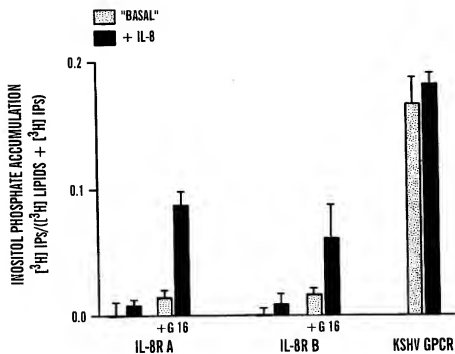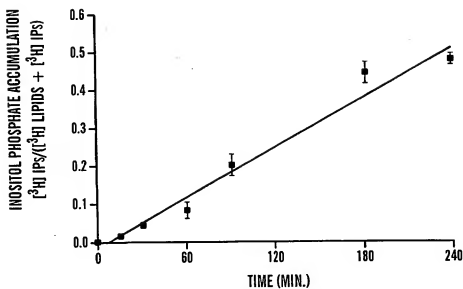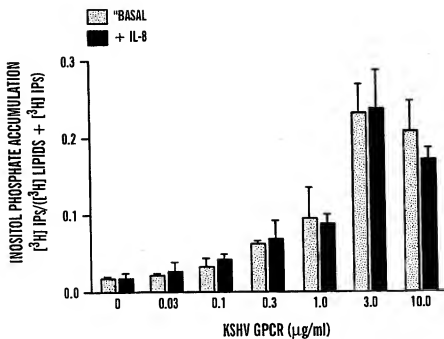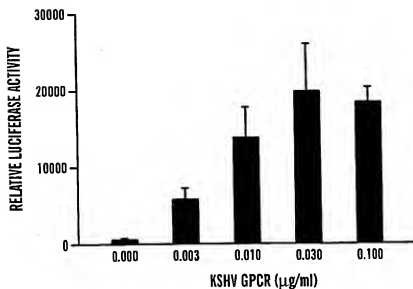receptor of human herpesvirus 8.

35

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8